

Implications of Some Genetic Control Mechanisms in *Neurospora*

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INTRODUCTION

Apologia

For the would-be writer of a review article, it can be sobering to ask whether the article is needed. On posing this question to myself, I realized that a number of very perceptive and, in some cases, extensive reviews have already appeared in print. These are listed below. I saw few ways to cover the same ground differently, and certainly no way to cover it better. It seemed more profitable to use this space as an opportunity (i) to examine a few of the special features of *Neurospora crassa* and closely related species that are likely to escape attention elsewhere and (ii) for some unabashed speculation about the evolutionary pressures that have shaped the control mechanisms extant in *Neurospora* and perhaps in other eucaryotes. This article is meant to be read with that in mind.

Books, Reviews, and Other Sources of Information

The following sources will be useful to those wishing to get abreast of the literature of *Neurospora*.

Fincham and Day (45). General fungal genetics textbook, with emphasis on *Neurospora*. Chromosome mechanics, mutagenesis, mapping functions, fine structure determination, extra-chromosomal inheritance, physiological genetics.

Davis (37). A critical review of metabolic organization and compartmentation of function in *Neurospora* and other fungi. Prevention of futile cycling by summation of anabolism and catabolism.

Marzluf (89). A valuable discussion of genetic regulation of metabolic pathways in fungi.

Perkins and Barry (103). A remarkable, comprehensive review of cytogenetics, meiosis and mitosis, and chromosomal aberrations; life cycle of *N. crassa* and related heterothallic and pseudohomothallic species. Contains genetic maps and tabulations of kinds of auxotrophs and morphological, color, and other kinds of mutants.

Perkins et al. (105). Natural history of the genus *Neurospora*. Taxonomy and chromosome homology of a large collection of isolates from nature.

Srb et al. (121). Genetics of development of the sexual apparatus.

Nelson et al. (98). Biochemical and morphological changes during conidium formation, especially as revealed by mutational dissection.

Schmit and Brody (116). Biochemistry and physiology of conidial germination.

Brody (20) and Scott (117). Effect of enzyme levels and metabolic pools on cell wall and membrane composition and on morphology.

Mishra (95). Various aspects of morphogenesis.

Bachmann and Strickland (12). An inval-

uable, comprehensive source of information, with work indexed by gene locus as well as by subject and author.

Bachmann (5-11). Updatings of Bachmann and Strickland (12).

Davis and DeSerres (38). Techniques used in doing research with *Neurospora*, with discussion of their theoretical basis.

"Stanford Neurospora Methods" (122). Some additional, practical techniques for working with *Neurospora*.

Perkins (102). Ascus dissection, especially techniques for working with unordered tetrads.

Brief Description of the Life Cycle and Natural History of *Neurospora*

Nutritional requirements. *Neurospora* can grow at essentially the maximal rate with only a carbon energy source, mineral salts, and one vitamin, biotin. The latter is absolutely required. The most readily used carbon energy sources are the monosaccharides glucose, mannose, fructose, and xylose and such disaccharides as sucrose, maltose, cellobiose, and trehalose. Disaccharides of exclusively animal origin, such as lactose, are used very poorly. Inorganic sulfate is a good sulfur source, though thiosulfate, methionine, taurine, cysteic acid, and many other compounds can be used instead. Phosphate is the only excellent phosphorus source (see, however, Phosphorus Acquisition System). Ammonium ion is the most commonly used nitrogen source, though nitrate ion, urea, various amides, purines, and a number of amino acids can be used instead (16, 109). Such inorganic ions as potassium, magnesium, and the usual trace elements are, of course, necessary, with iron acquisition being especially critical under certain conditions (67). The pH range for growth is about 4.0 to 7.5, with growth being poor at either extreme. Vegetative growth is optimal at about 30 to 35°C, though the mold can grow at temperatures as high as 42 and as low as 5°C. *Neurospora* is an absolute aerobe and is considered completely nonpathogenic to humans, animals, and plants.

Ploidy, chromosome number, and genome organization. *N. crassa* is normally haploid during growth. The sole diploid cell in the life cycle is the zygote nucleus formed by fusion of two haploid nuclei. The diploid nucleus is promptly reduced to haploid products by meiosis. In the haploid state, the organism has seven chromosomes, each having, on the average, slightly more deoxyribonucleic acid (DNA) than does *Escherichia coli* (68) or, by more recent measurement, slightly less (R. Krumlauf and G. A. Marzluf, Biochemistry, in press). Some

90% of the DNA is present in unique sequences (Krumlauf and Marzluf, in press; see also references 21 and 41), 2% of it consists of foldback elements that renature very rapidly even in dilute solution, and 8% is repetitive. The repetitive DNA is not organized in the short interspersed sequence pattern that has been established for many other eucaryotes, but as sequence(s) that are at least 10,000 nucleotide base pairs long and that may be much longer. Very little of the single-sequence DNA is contiguous with the repetitive DNA. It appears that most of the repetitive DNA can be accounted for as ribosomal DNA. The ribosomal DNA which codes for the 17S, 5.8S, and 26S ribosomal ribonucleic acids (RNA) exists in the genome in multiple, apparently identical, copies, which include transcribed and untranscribed spacer regions; the transcribed region and the total repeat unit have been cloned into plasmid pBR322 (48; S. J. Free, K. Driftmier, and R. L. Metzenberg, manuscript in preparation). The ribosomal DNA repeat unit has a size of roughly 6 megadaltons (about 9,700 nucleotide base pairs). If all, or nearly all, of the repetitive DNA can be accounted for by this ribosomal DNA repeat unit, that is, 8% of 2.7×10^7 nucleotide base pairs, there could be as many as 220 of these repeats. The actual figure appears to be closer to 185 (R. Krumlauf and G. A. Marzluf, personal communication). Surprisingly, the 5S ribosomal RNA is not coded by this repeat unit, as it is in yeast (130), but is embedded in DNA with many different kinds of flanking sequences (48). Earlier workers failed to find histones in *Neurospora*, but later investigators who used the wall-less mutant (see below) to isolate chromatin and took precautions to avoid proteolysis have found proteins that apparently correspond to H2A, H2B, H3, and H4 (70), and H1 as well (60). The chromatin of *Neurospora* is organized into nucleosome-like structures that are smaller than those of higher eucaryotes but that seem otherwise rather similar (99).

Cell types. *Neurospora* advances along a solid surface or grows in liquid medium almost exclusively as a mycelium composed of tubular hyphae, and it is in this form that almost all assimilation and mass increase occurs (see How *Neurospora* Grows for further information). On a surface, behind a growing front, two different kinds of specialized hyphae may be found. One of these, the aerial hypha, is the bearer of asexual spores called *macroconidia* or simply *conidia*. Each conidiophore will carry a long, linear string of the conidia (98). The conidia are produced in great numbers; much of the mass of mycelium is apparently converted in stationary phase to these asexual spores. The number of nuclei per

conidium is variable, the average being roughly 2.5 under the most common laboratory condition (4, 71). These conidia are very resistant to killing by desiccation and can be stored indefinitely with silica gel. At high humidity, however, they survive only 1 or 2 months at room temperature. Conidia are completely indifferent to freezing, and a slant with abundant conidia can be kept many years in an ordinary deepfreeze without loss of viability. *Neurospora* conidia, like those of most fungi, are quite light and can be carried by winds over great distances. On landing upon a suitable nutrient medium, a conidium germinates after about 2 to 5 h, resuming the hyphal growth form.

A second sort of asexual spore is also formed from hyphae, but it is extruded directly from stationary-phase mycelial cells, rather than being formed from aerial hyphae. These are the *microconidia*, which are uninucleate and (obviously) smaller than macroconidia. They are generally produced only in relatively small numbers. Strains are available that make no ordinary conidia at all but that still make microconidia normally. Microconidia germinate erratically and are little used in research. Their biological role is less obvious than that of the other cell types.

N. crassa is heterothallic. It exists in two mating types, designated *A* and *a*, and no mating can occur unless both are present. The two alternatives are determined by a pair of alleles at a unique locus, the mating type locus. The two mating types are phenotypically indistinguishable to us; only *Neurospora* can tell them apart. The mating types should not be confused with sexes. If a female is defined as the sex that contributes most or all of the cytoplasm to the zygote, either mating type can act as either male or female. The way in which this can be controlled is particularly simple. If mating type *A* is inoculated onto the medium first and allowed to grow several days, it will be the female. The later arrival on the scene will function as male. The reason for this becomes apparent when the structures involved in mating are examined.

If *Neurospora* is inoculated onto a solid medium that is marginally deficient in certain constituents or has nitrate ion as the sole nitrogen source (136), some of the hyphae curl up into a specialized form, the ascogonium, which is surrounded by sterile hyphal tissue. This female structure, which is just visible to the naked eye, is the *protoperithecium*. In its early stages, it looks very much like a coil pot in the process of construction. The tip of a specialized ascogonial hypha is further differentiated into a *trichogyne*, a structure which projects from the protoperi-

thecium. When a conidium, microconidium, or even a piece of mycelium of the opposite mating type lands on the trichogyne, a donor nucleus (male) is conducted into the ascogonium, where it meets the resident (female) nucleus. The two do not fuse right away. Both divide in concert, and a number of cells are generated that contain one nucleus of each mating type. When the nuclei of the two mating types do fuse, meiosis proceeds promptly. The four meiotic products each undergo a mitotic division to give four pairs of sister nuclei, and each of these eight is delimited to become an *ascospore*, or sexual spore. (The nuclei in the ascospores themselves divide to give 16 nuclei, but this is usually without genetic consequence.) The maturing, football-shaped, black ascospores from each meiotic event are contained in a sack, the ascus. The restriction on the movement of the meiotic spindles in the developing ascus is such that the ultimate order of the spores in the ascus is significant (for details, consult Fincham and Day [45] or any standard genetics text). The dimensions of an ascus are about 20 by 200 μm , and there may be several hundred in a mature fruiting body, a *perithecium*. The spores may be removed individually from an ascus, preserving the order; the ascus may be collected with the spore order randomized; or the spores be collected after they have been spontaneously shot from the perithecium and randomized with others from the same or other perithecia. Useful data can be obtained from ascospores collected in any of these ways.

The ascospores are, of course, products of a sexual process, and as such they are likely to enjoy whatever advantages may be conferred by recombination of parental traits. The cardinal twin properties of ascospores that probably determine the ecological niche of *Neurospora* are their dormancy and their resistance to heat. Ascospores respire extremely slowly and can lie dormant for years at ambient temperature and humidity—much longer than conidia, though they survive freezing poorly. Unlike other cell types, the ascospores do survive heat shocks of 60°C and more, even in the hydrated state.

Indeed, this is one of two ways in which dormancy is broken. The other is exposure to even very low concentrations of certain volatile ethers, particularly furfural (42, 124). But furfural ($\text{C}_5\text{H}_4\text{O}_2$) is a major product of dehydration formed when xylose or "wood sugar" ($\text{C}_5\text{H}_{10}\text{O}_5$) is heated. Thus, either heat or the material resulting from heating woody materials will trigger the germination of ascospores. It is not surprising, then, to find that *N. crassa*, which is ordinarily inconspicuous or unobservable in na-

ture, becomes prominent right after a forest or brush fire, a volcanic eruption, or the burning of a field of cane stubble (105). It occurs in other places subjected to intermittent heat: bakeries, lumber kilns, and bagasse presses. The common pattern seems to be that dormant ascospores are activated and regenerate the mycelial state. The mycelium grows on whatever vegetable exudates are present, and conidia are formed; these are dispersed by air currents. They may initiate new vegetative growth on a suitable substrate, or they may fertilize protoperithecia in some neighboring clone which happens to be of the opposite mating type. In the latter case, the next generation of ascospores may lie dormant for years before another heat shock starts the cycle anew. The natural history of this mold explains much about its behavior as it is known in the laboratory, including its ability to spread rapidly over an essentially axenic surface, its partiality to sugars, such as sucrose, glucose, and xylose, and its elaboration of disaccharases, cellulase, and α -amylase.

Miscellaneous Underexploited Possibilities

The ease of selecting mutants and doing genetic analysis and the economies of keeping large numbers of strains of *Neurospora* have received adequate attention. These must be weighed against some of the difficulties of working with filamentous organisms, which are not to be ignored. A number of these are rather easily circumvented; for example, the conidia or very young mycelia of *Neurospora* are easily pipetted, and if they are plated onto medium containing sorbose and small amounts of other monosaccharides (18, 125), they develop into compact colonies of intensely branched mycelia. These can be easily counted, replica plated (83), scored for the presence of extracellular enzymes, etc.

In several cases, the filamentous cell form is turned to advantage. An extremely effective way of selecting auxotrophs is filtration-enrichment, in which mutagenized conidia are suspended in minimal medium and filtered at intervals through cheesecloth or glass wool (2a, 31, 139, 141). The surviving prototrophs become tangled in the filter and are removed, whereas dead spores and auxotrophs pass through. Finally, the suspension is plated onto the nutrient for which auxotrophs are desired. The filamentous structure of hyphae allows microelectrodes to be inserted so that electrophysiology can be done on an organism in which transport mutants can also be selected (119). Finally, solutions and even organelles can be microinjected into hyphae of *Neurospora* (137, 138).

One of the oddest strains of this genus is one that not only does away with hyphal structure but does away with cell walls as well. This mutant strain, called "slime" because of its superficial resemblance to the plasmodium of a slime mold, actually owes its morphology to at least three mutations (43). It will grow in gently shaken isosmotic liquid medium as multinucleate protoplasts and can be kept in exponential growth indefinitely (140). Unlike wild-type *Neurospora*, which has a tough cell wall, slime is very easily broken by shearing, sonication, or detergents. Thus, it lends itself extremely well to isolation of cell membranes (17, 115), nuclei (70, 126; R. E. Nelson, R. Totten, and R. L. Metzenberg, manuscript in preparation), and high-molecular-weight DNA (S. J. Free and R. L. Metzenberg, unpublished observations). Slime can be fused with liposomes (94), and there is reason to think it can be fused with other kinds of cells. Despite anecdotal warnings about it being hard to grow and keep, I have found that it survives more than 1 month in liquid culture at room temperature and more than 1 year on a slant at 4°C, and it can be kept indefinitely at -70°C in dimethyl sulfoxide (35). One drawback of slime is that mutant genes of one's choice are not readily crossed into it. This problem can be overcome by use of certain techniques (97).

One technical advance that is certain to extend the experimental possibilities is the very recent development of an efficient transformation system. The *Neurospora* dehydroquinase gene, cloned into *E. coli* plasmid pBR322, has been successfully reintroduced in *Neurospora* mutants lacking that activity (M. E. Case, M. Schweitzer, S. Kushner, and N. H. Giles, personal communication).

SOME THEORIZING

How *Neurospora* Grows

Filamentous fungi have a distinctive way of obtaining food. Their mode of feeding merits the attention of a physiological geneticist because it is interesting in itself and because it shapes genetic control mechanisms in subtle but important ways. Consider the idealized case of exponential growth of a branched, tubular, coenocytic structure, such as *N. crassa*, and contrast it with that of a more familiar organism, such as *E. coli*. In the latter, a single cell will give rise to a culture which doubles in mass and number at some characteristic rate, perhaps every 25 min. This will continue until some nutrient becomes limiting or until excreted waste products slow the rate of increase—in other words, until the environment produced by one bacterium be-

comes part of the environment of its neighbor. A nonmotile bacterium landing on a rich solid medium and incubated at a suitable temperature will initially grow in much the same way as in liquid medium, but limitation of space and nutrients will occur much sooner. Whereas the rate of growth for the first few divisions might easily suffice to give a bacterial mass equal to the earth's mass in 2 days, the actual size of the colony after 2 days might be only a few millimeters in diameter. Clearly, *E. coli* can physically and biochemically occupy space very rapidly in a liquid medium, but much less so on a solid substratum.

Compare this with a conidiospore of *Neurospora* germinating on a liquid or solid medium. Dissolved nutrients are transported into the cell, and new wall material is laid down to give a pipeline-like cylindrical structure, a hypha. Septa are soon laid down, but they are perforate, and nutrients and organelles, including nuclei, are carried from one compartment to the next. As the two closed ends of a hypha are extended outward, the mass of the organism increases and may double about every 2.5 h (36, 85), or about one-fifth as rapidly as *E. coli*. With the increase of mass comes an increase in number of nuclei and in capacity for assimilation and for biosynthesis. The resulting increase in turgor pressure due to the number of osmotically active particles can be relieved as long as the rate of linear extension at the ends of the cylinder also increases approximately exponentially.

Obviously, this cannot continue for very long. When the production of osmotically active particles exceeds the capacity to elongate at two ends, a third end—a branch—appears, and shortly a highly branched, mycelial structure is formed. The rate of linear extension of a mycelial front of wild-type *Neurospora* can be as great as 0.6 cm/h (105). Although there are many conditions, such as nonoptimal temperature and genetic constitution, that will markedly diminish this rate (38, 57), the rate of advance of a mycelial front on a solid medium usually depends surprisingly little on nutrition. It is the density of branching behind the front that varies dramatically (56, 57). Hence, on a rich, mediocre, or poor medium, *Neurospora* can generally push forward a mycelial front at a rate of 10 to 14 cm/day. The selective value in being able to do this requires no comment. It can be expected that in colonizing fresh territory, the organism will be actively projecting itself into environments that change rapidly in space as well as in time. At the front and behind it, the territory staked out by the mycelium is exploited for foodstuffs. This involves the secretion of hydrolytic enzymes into

the medium and into the periplasmic space as well as the synthesis of permeases. Since this mycelium is usually genetically as well as cytologically a single organism (105), this does not amount to group selection. Because the mode of feeding is essentially one of rapid advance and then engulfment, it is not surprising that *Neurospora* can adapt to changes in environment with radical changes in the synthesis of certain enzymes. These include enzymes for the biosynthesis of building blocks, but also hydrolases and permeases needed for the acquisition of carbon, nitrogen, phosphorus, and sulfur sources.

Some Problems of Large Cells

One physical parameter of the cell that certainly must be of selective importance is the osmotic pressure; yet this has usually been neglected by cell biologists. It may be useful to consider a simple model of a cylindrical cell, say 10 times the diameter of *E. coli*. The force exerted at the round end plates will be 100 times that of the bacterium, provided the two are at the same osmotic pressure. There will, however, be 10 times as much wall material around the circumference of the larger cell to resist the tearing force exerted at the ends. The net effect is that the tendency of the cell to rupture in a given hypotonic medium should be proportional to the first power of the cell dimensions. It seems reasonable, then, that a large cell will be under especially strong selection for holding its content of osmotically active particles within reasonable bounds. I will argue that at least two common stigmata of *Neurospora* and some other well-studied eucaryotes can be understood in this light: (i) multifunctional polypeptides capable of "channelling" metabolic intermediates and (ii) positive control of gene expression.

The multifunctional polypeptides may help solve two problems at once, as will be developed below (see The *aro* Anabolic Enzyme Aggregate). Reducing the number of osmotically active polypeptide chains may be to some degree valuable in itself, and reducing the need for large, cell-wide pools of free intermediates is likely to have a still greater effect. The prevalence of these multifunctional polypeptides in eucaryotes has been pointed out by Stark (123).

The appropriateness of positive control of gene expression for holding down intracellular osmotic pressure during, say, exponential outgrowth of a fungal hypha is unproven but not unreasonable. By positive control, one means that the natural condition of structural genes is one of inactivity and that positive action of a specific macromolecule, which might be provisionally called an "expressor," is needed to pro-

voke the system to the active state. Presumably, this means stimulation of specific transcription and implies that the expressor must bind to some site on DNA immediately upstream from the structural gene, perhaps in the promotor region. The genome size of *Neurospora* has been variously estimated as about 10 or 5 times that of *E. coli* (68; Krumlauf and Marzluf, Biochemistry, in press). (The haploid genome size of humans is roughly another two orders of magnitude greater.) *E. coli* has a volume of very roughly 10^{-15} liter; it has roughly one structural gene of each sort (or $1/\log_2$, if it is dividing exponentially on a poor medium). The concentration of each gene in the cell is therefore about 2×10^{-9} M (133). In *Neurospora*, both the diameter of the hyphae and the length of each cell compartment governed by a nucleus are variable, but the general dimensions are in the range of 10 times those of *E. coli*, to give a cell volume about three orders of magnitude greater and a concentration of each structural gene of perhaps 2×10^{-12} M. Yet the number of different structural genes in *Neurospora* is likely to be greater, perhaps the 5- to 10-fold greater suggested by the genome size. It is easy to see intuitively that for an organism with a relatively large number of individual genes but a low molar concentration of each gene, it demands less regulatory vigilance to have each gene inherently inactive except when it is specifically called upon. The mechanism of negative control, which is common in *E. coli* but which is, at best, rare in *Neurospora*, requires the regulatory macromolecules to actively hold most genes in check most of the time. There is probably some upper limit on the genome size for which negative control is practical, and that limit might be in the range between 5×10^6 and 1.5×10^7 base pairs—the region of the discontinuity between procaryotes and eucaryotes.

Looking at the genome size from another viewpoint, it is generally assumed that regulatory mechanisms are much more complicated in eucaryotes than in bacteria and that this will be reflected in proportionately more different kinds of regulatory macromolecules. If negative control were the rule in these complicated cells, each negative controller (repressor) would need to be present at a concentration at least comparable to that of the intracellular average of lactose repressor, or about 2×10^{-8} M (76). The only alternative to high total concentrations of repressor would be to create repressors with substantially lower dissociation constants than those occurring in bacteria, or to settle for a lesser degree of control. But the lactose repressor and other known repressors have very likely

already been selected for the lowest binding constants consistent with noncovalent forces between protein and nucleic acids and with being at the same time a binding that can be reversed within reasonable time by an external metabolic signal (53). Assume for argument that there are about 1,000 regulated systems in *E. coli*, most of them repressed in a typical cell, and that there are 10 times as many kinds of regulatory macromolecules in *Neurospora*, each at the same concentration as the lactose repressor of *E. coli*. The total concentration of these hypothetical repressors in *Neurospora* would then be of the order of 2×10^{-4} M. If an average repressor molecule has a molecular weight of 10^5 , this would demand a protein concentration of 20 mg/ml due to repressors alone and a direct osmotic pressure of about 4 torr. Neither of these figures seems impossibly large; however, the osmotic pressure attributable to the protein itself is only the tip of the iceberg. At intracellular pH values, these proteins will not generally be at their isoelectric points. Most will be polyanions, carrying highly variable numbers of negative charges due to the excess of glutamic and aspartic acid residues over those of basic amino acids. For a protein with a size of 10^5 daltons containing 10^3 amino acids, a net of 50 negative charges at intracellular pH values is not unreasonable. Each of these will be neutralized by some small cation, generally K^+ . Most evidence indicates that these cations are sufficiently free to be fully osmotically active. Thus, if *Neurospora* were regulated mostly or entirely by negative control, we could expect additional intracellular concentrations of particles of the order of 10^{-2} M for that reason alone, and with it an osmotic pressure increment of perhaps 200 torr.

These order-of-magnitude estimates are based on the supposition of about a 1,000-fold difference between a fully repressed and fully depressed system, as with the lactose operon. Yet there are indications that eucaryotes are capable of a higher range of metabolic and epigenetic control than are bacteria—more than 10,000-fold for the alkaline phosphatase system of *Neurospora* (81) and probably that much or more for control of tissue-specific protein synthesis in differentiated organisms (113). If control ratios of that order of magnitude are required in the economy higher organisms, negative control as a general mechanism may pose insuperable problems.

By contrast, it is easy to see how a positive control substance can control expression of genes over any desired range. Furthermore, it can be accomplished with very much smaller concentrations of protein than would be neces-

sary with repressors which effect negative control. This does not require postulating lower dissociation constants between expressors and nucleic acids than would be the case with repressors. Rather, it proceeds from the fact that, with negative control, one must cover the range of 100% turned on (no active repressor), to say, 0.1% turned on (99.9% association of repressor and target) in the case of the lactose system. The latter naturally requires relatively high concentration of the repressor. With positive control, one may cover the range of 0% turned on (no active expressor) to, say, 70% turned on (70% association with target), to give essentially an infinite multiple of expression. Covering 99.9% of the targets in a negative control system requires an effective repressor concentration of 1,000 times the dissociation constant. Covering 70% of the targets in a positive control system requires an expressor concentration of only about 2.3 times the dissociation constant. It seems reasonable that a small to moderate reduction in the maximum rate of transcription might not be too high a price to pay for an unlimited range of expression, achieved at an actual reduction in biosynthetic and osmotic costs. This may be all the more true of a large cell that is osmotically more vulnerable than a small one. The argument should apply even more strongly to cells of higher eucaryotes, in which the genome size and, quite likely, the complexity of control are one to two orders of magnitude larger than those of *Neurospora*. It pertains most of all to animal cells, in which no strong cell wall is present.

ANABOLISM AND CATABOLISM: CONTROL MECHANISMS AS REFLECTED IN FOUR GENE-ENZYME SYSTEMS

In general, synthesis of most anabolic enzymes in *Neurospora* seems to be either constitutive, as in the *aro* system (to be examined below), or under a relatively limited degree of control (see, for example, reference 25). Instead, strong control is often exerted via feedback inhibition. An outstanding exception, in which both feedback inhibition and positive control of enzyme synthesis combine to effect a high degree of regulation, is seen in the case of leucine biosynthesis (61, 108). In this system, the first enzyme private to leucine biosynthesis is under feedback control by leucine. The product of that enzyme, α -isopropylmalate, apparently interacts with a positive control factor produced by the *leu-3* gene to provoke expression of structural genes for two subsequent steps in the pathway. In the absence of either α -isopropylmalate or *leu-3*⁺ product,

these genes are not expressed. Because this interesting system has been discussed in detail rather recently by its principal proponent (see reference 107), it will not be examined further in this section.

Quinic Acid Catabolic System

One of the most extensively studied gene-enzyme systems in any eucaryote is the group of three highly inducible enzymes that catalyze the first three steps in the catabolism of quinic acid. This compound is widely distributed in plants, being abundant in such diverse material as quinine bark, maize, coffee, and plums, so it is not surprising that *Neurospora* is able to use it adaptively as a carbon and energy source. The three enzymes, quinate dehydrogenase, 5-dehydroquinate dehydratase (catabolic dehydroquinase) and dehydroshikimate dehydratase conduct, respectively, a dehydrogenation and two dehydrations to convert quinic acid to protocatechuic acid (Fig. 1). The three enzymes are coordinately induced by quinic acid (32, 33). Other enzymes, which will not be considered further, allow protocatechuic acid to be oxidized and brought into the central catabolic pathways.

The three highly quinate-inducible enzymes are coded by three structural genes designated, respectively, *qa-3*, *qa-2*, and *qa-4* for the three successive enzymes in the pathway (54; H. W. Rines, Genetics 60:215, 1968). The order of these structural genes on the chromosome, namely *qa-3*, *qa-4*, and *qa-2* (30), is different from that of the enzymatic steps. Numerous missense mutants have been found in all of these genes, but no polar or other nonsense mutants have been found. Currently, there is no way of deciding whether the three structural genes give rise to a single transcript, or a transcript that is cut into cistron-sized pieces before translation, or whether three messenger RNA molecules are

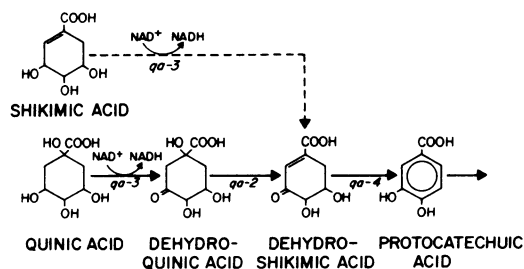


FIG. 1. Pathway of catabolism of quinic acid. The three structural genes which encode the enzymes for this part of the pathway are indicated under the arrows. The further oxidation of protocatechuic acid is under a different system of control and is not considered in this article.

transcribed in the first place, each from its own promoter. Whether or not the enzymes are made from a single messenger RNA, it seems clear that the enzymes are separate polypeptides, with no tendency to exist as an aggregate.

Just proximal to the centromere from *qa-3* is the control gene, *qa-1*. This makes a diffusible product which is viewed as being activated by quinic acid and which is necessary for the expression of the three structural genes. In other words, it is a positive control element. The *qa-1* product is almost surely a protein, since alleles with temperature-sensitive expression have been found, and different defective alleles can complement to give a functional product (29). It is perhaps easiest to imagine the *qa-1* product exerting its action by binding to a target region of DNA between *qa-1* and *qa-3*. No mutations that confer independence from the *qa-1* product have been found in this hypothetical target. While it continues to seem likely that such a target region of DNA exists and will ultimately be marked by mutations or by suitable *in vitro* binding studies, other possible modes of action cannot be strictly ruled out. For example, it is possible that even in the absence of a functioning *qa-1* product, transcription begins at one or more promoters for the gene cluster or the individual genes but that in the absence of the positive control element from *qa-1*, this incipient transcription terminates or awaits a signal to continue. It is not really known for sure whether control is transcriptional or posttranscriptional. One level of control that can be excluded is the posttranslational one; isotope transfer experiments have shown that at least two of the enzymes, and presumably all three, are formed *de novo* after induction with quinic acid (74; W. R. Reinert and N. H. Giles, *Genetics*, in press).

To state that the *qa-1* product is freely diffusible between nuclei is to paper over certain complexities. Pleiotropic null *qa-1* mutants do complement mutants in the *qa-3*, *qa-4*, and *qa-2* loci to give heterocaryons that grow on quinic acid as a carbon source, but they do so at very different rates. This allows the mutants to be placed in two sharply bimodal categories of fast and slow complementers, designated *qa-1^F* and *qa-1^S* (74, 131; Rines, *Genetics* 60:215, 1968). This might be dismissed as some complicated, minor phenomenon if it were not for two facts: the two classes of pleiotropic null *qa-1* mutations map in separate, nonoverlapping parts of the gene (29) and *qa-1^S*, but not *qa-1^F*, mutants can give rise to secondary intragenic mutants that are pleiotropic constitutive (*qa-1^c*) for expression of the structural genes (131). These *qa-1^c* mutants, which can also be isolated from *qa⁺* strains

by a screening technique (101), are able to make the enzymes of quinate catabolism in the absence of the inducer, quinic acid. A natural way to interpret this is to suppose that the region of the protein affected by the *qa-1^S* mutations is the domain that binds to the target region on the DNA and that other kinds of changes in the same region (*qa-1^c* mutations) can cause the protein to bind to the DNA even without the help of quinic acid (74). It would seem possible however, that the region altered in the *qa-1^F* mutants is the one which binds DNA and that no second-site mutation can give a functional product independent of quinic acid activation.

Whatever the biochemical roles of the *qa-1^F* and *qa-1^S* regions, it remains rather hard to explain their complementation behavior on the basis of a single, diffusible *qa-1* product. Complementation studies have usually involved one of these control mutants in a forced heterocaryon with a structural gene, such as *qa-2*, mutant. For additional clarity, let us momentarily represent the two kinds of nuclei in a heterocaryon as *qa-1^{-(F or S)} qa-2⁺* and *qa-1⁺ qa-2⁻*. A paradox then asserts itself. If all that is needed for the function of *qa-2⁺* is for a molecule of the *qa-1⁺* product (active for both *qa-1* functions) to diffuse over to the *qa-1⁻ qa-2⁺* nucleus, then one cannot readily explain why *qa-1⁺* molecules arrive slowly and incompletely at *qa-1^{-(S)} qa-2⁺* nuclei but arrive rapidly and with full effect at *qa-1^{-(F)} qa-2⁺* nuclei. The explanation favored by the investigators is that the *qa-1^{-(S)}* product should not be thought of as functionless, but rather as being able to form mixed multimers with the *qa-1⁺* product thus causing negative complementation, but that this impairment of the *qa-1⁺* product does not occur in combination with *qa-1^{-(F)}* mutants. This explanation is certainly in accord with the data, but it seems to beg the question of why all mutants in one segment of a cistron are capable of negative complementation and why all mutants in the other segment are not. One is almost inclined to consider an alternate model, involving two cistrons and two proteins, one easily diffusible and the other much less so. Ordinarily, the failure of certain *qa-1^S* and *qa-1^F* mutants to complement would allow them to be defined as being in the same cistron, and hence the alternative of two *qa-1* genes could be rejected (29). However, the failure or near-failure of one of the kinds of mutants to complement in heterocaryons with structural gene mutants that are unquestionably in different cistrons weakens the credibility of the *trans* part of the *cis-trans* test. The same problem of a gene product that is poorly diffusible between nuclei arises in trying to assess the

degree of dominance of *qa-1^c* mutants in a heterocaryon (29, 131).

Many of these questions may soon be resolved by biochemical approaches. Vapnek et al. (132) and Kushner et al. (79) have reported isolation of derivatives of plasmid pBR322 that carry at least the *qa-2⁺* gene in a form that can be expressed in *E. coli*. The isolations involved transformation of an *E. coli* mutant lacking dehydroquinase (anabolic) with plasmids carrying *Hind*III or *Pst*I restriction fragments of *Neurospora* DNA. Expression of the catabolic dehydroquinase of *Neurospora* in *E. coli* transformants allowed the bacteria to grow without supplementation with aromatic amino acids. The molecular weight, heat stability, fractionation behavior, and immunological specificity of the enzyme were shown to be those of the *Neurospora* catabolic dehydroquinase, not those of the anabolic *E. coli* enzyme. It is not yet known whether other structural genes of the cluster are carried in these hybrid plasmids, but if they are, they are not expressed (2). Since either the *Pst*I or the *Hind*III fragments give rise to about the same expression of the *qa-2* gene in either orientation with respect to plasmid DNA, it is very likely that the RNA corresponding to this gene is transcribed from a *Neurospora* DNA promoter, though not necessarily the same promoter that is used in *Neurospora*. The original DNA from which the *Neurospora* restriction fragments were prepared was from a *qa-1^c* mutant, but it is doubtful that the *qa-1* is present in at least the smaller of the two cloned inserts; and even if it is present, it is unlikely that *qa-1^c* product is formed. Instead, it seems that *E. coli* RNA polymerase can initiate transcription on the cloned fragment independent of the *qa-1* product, though there is no evidence that it does so at the normal promoter. If, however, the *qa-1* product is actually present and causing expression of *qa-2*, then presumably *qa-3* and *qa-4* are also carried on the plasmid, since they map between *qa-1* and *qa-2* (30). The question of whether *qa-1*, *qa-3*, and *qa-4* are present should be more easily answered now that a transfor-

mation system has been developed (see Introduction). In addition, access to pure DNA carrying the putative binding site for the *qa-1* product should greatly facilitate attempts to isolate that protein and study its mode of action. It should also make it possible to reconstruct an in vitro transcription-translation system to extend our understanding of this regulatory system to the molecular level (74).

The *aro* Anabolic Enzyme Aggregate

The biosynthesis of aromatic amino acids in *Neurospora* starts with erythrose 4-phosphate and phosphoenolpyruvate. It proceeds through seven enzymatic steps to arrive at chorismic acid, the final precursor before the common pathway branches toward the individual aromatic amino acids (Fig. 2). The seven intermediates are identical with those in *E. coli*, but the enzymatic machinery is organized in a very different way. In *E. coli* and in at least five other procaryotes, each enzyme is a separable molecular entity, and in all cases investigated, each was coded by a distinct structural gene (14). In *Neurospora*, steps 2 through 6 are part of a multienzyme aggregate that is, in turn, coded by five very discrete, tightly-linked regions which are almost certainly contiguous (26, 55). Both the genetics and the biochemistry of this gene-enzyme cluster have been intensively studied. Mutations in particular regions of the gene cluster give rise to missense mutants that are missing one enzyme or another, and generally these will complement with missense mutants that perturb some other activity, as would be expected of classical cistrons. Polar mutations have also been found. The polar mutations may be placed in a genetic order by mapping; the mutants may also be placed in a strict physiological order by which of the five enzymes survive disruption by the polar mutation—that is, which ones are upstream from the mutation. The rub is that the two orders are discordant. The genetic order, starting from the presumed promoter end, is *aro-2*, -4, -5, -9, -1. But there are pleiotropic mutations that map in the short region between

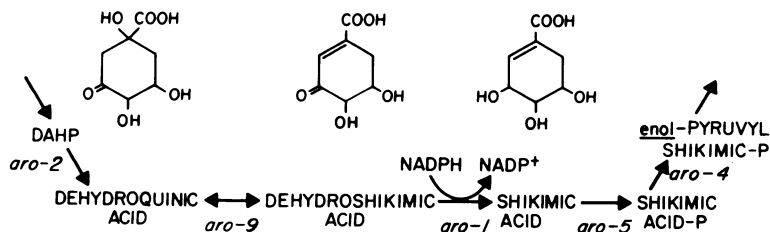


FIG. 2. Part of the pathway for biosynthesis of aromatic amino acids. The five steps catalyzed by the multienzyme aggregate are depicted, along with the five "cistrons" that seem to correspond to the activities.

aro-2 and *aro-4* missense mutations and destroy the activities corresponding to *aro-2*, -5, -9, and -1 while sparing *aro-4* (Fig. 3).

There are other anomalies. For example, there is a polar, apparently nonsense-type mutation in *aro-9* which obliterates the activities coded by *aro-9* and *aro-1* (27). Plating the mutant on minimal medium results in isolation of some first-site revertants. At least one of these revertants appears to be the result of converting the nonsense mutation in *aro-9* to a missense mutation. This revertant does not regain any *aro-9* activity (anabolic dehydroquinase), but it does regain *aro-1* activity (dehydroshikimate reductase). Since the anabolic dehydroquinase can be substituted by its catabolic counterpart, the strain is a prototroph. The surprise is that the restored *aro-1* activity is highly temperature sensitive—even though there is apparently no mutation in that “gene.”

This entire situation challenges our understanding of what a cistron is. Ordinarily, we would like to think of a cistron as a genetic entity that codes for a biochemical entity. (i) The genetic entity is such that any two missense mutants in different cistrons will show complementation in *trans* configuration and will show the mutant phenotype when they are tested in *cis*. Any two mutants altered in the same cistron will fail to show complementation in *trans* (except for allelic complementation, which typically gives a product with abnormal kinetic behavior or thermal stability or both). A mutation in one cistron will have no structural effects on enzymes coded by other cistrons. (ii) The biochemical entity is usually thought of as a covalently continuous polypeptide, running from an amino ter-

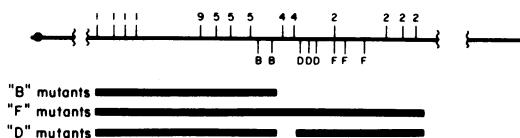


FIG. 3. Abbreviated genetic map and complementation map of the *aro* region of *Neurospora*, abstracted from information in Giles et al. (55) and Case and Giles (26a). Polarity of transcription is inferred to be right to left. Representative missense mutants that lack a single enzymatic activity and complement with mutants lacking any other single activity are indicated by the numbers 2, 4, 5, 9, and 1. “Pleiotropic” mutants lack two or more activities and fail to complement two or more kinds of mutants that lack single activities; these are indicated on the genetic map by letters B, D, and F (complementation types A, C, and E are omitted for simplicity). Pleiotropic D mutants, which occur just downstream from the region defined by simple *aro-2* mutants, complement only the simple *aro-4* mutants.

minus to the free α -carboxyl group. In the *aro* system, there are five genes that sometimes contradict the accepted rules of intercistron complementation. There are also five assayable activities that are physically so tightly associated as to be possibly a continuous polypeptide carrying all five activities. How do we decide whether there are five cistrons or one? How do we decide whether the enzymatic activities are held together with something other than peptide bonds? The answers are not yet clear. The genetic evidence cited above seems to mean that certain of the pleiotropic mutations map as if they were in a single gene but have effects on the properties of “enzymes” coded by other “genes” in the cluster. The most obvious medium of propagation of these changed intensive properties is a continuous polypeptide chain. When one tries to examine the physical structure of the pentafunctional enzyme complex, a different kind of uncertainty arises. There is general agreement that the overall structure of the entity is that of a dimer of some sort, with the total molecular weight being variously estimated as 231,000, 300,000, and 330,000 (22, 50, 86). According to one view, each of the monomers may in turn be a very tight noncovalent aggregate of a number of different polypeptide chains, each being coded by a more or less classical cistron (22, 73). Jacobson et al. (73) and Burgoyne et al. (22) have found a number of conditions under which at least some of the activities can be liberated from the larger complex as proteins of lower molecular weights. On the other hand, each of the monomers can also be viewed as a single polypeptide (50, 86). Gaertner and Cole (50) and Lumsden and Coggins (86) consider it likely that the pentafunctional enzyme complexes isolated by Giles and co-workers (22, 73) have been nicked by proteases, though remaining active. By a combination of the use of protease inhibitors, the use of affinity columns, and working speedily, Gaertner and Cole and Lumsden and Coggins groups have prepared enzymes which do not appear to be dissociable to anything smaller than half the full-sized enzyme. Gaertner and Cole (50) suggest that not only is the *aro* complex a dimer of two simple chains but the unit coding for it should be thought of as a single gene. They call this a “cluster gene,” as opposed to the earlier “gene cluster,” to emphasize its singleness. The debate over the *aro* complex of *Neurospora* is not a unique one. The same conceptual and experimental uncertainties are being thrashed out in several other eucaryotic systems—perhaps most thoroughly in the *his4* system of yeast (15a). The same enzymes of the *aro* complex of

Neurospora are also found as a complex in a variety of other fungi (1) and in *Euglena* among photosynthetic organisms (13). Therefore, it seems likely that some advantage must accrue to eucaryotic organisms as a result of the existence of this complex. Giles et al. (55) have suggested that one function might be to channel anabolic intermediates through the complex to prevent their entering the competing catabolic pathway. The correlation between the possession of a pentafunctional *aro* complex and the possession of a degradative pathway for quinic acid is not strong, however (15). Other workers (51, 134, 135) have done kinetic studies on the complex, and this work reveals other possible advantages of having the activities present in a single molecule. We can imagine three ways in which such an aggregation of activities might lead to more rapid catalysis with a given concentration of enzymes and of substrates. First of all, there could be effects of the maximum velocity (V_{max}) of the enzymes achieved at saturating substrate concentrations. Second, there could be effects on lowering the K_m , so that a given finite substrate concentration would give a velocity (v) that is not so small compared with V_{max} . Finally, if the contents of a cell are not being instantaneously mixed, the close proximity of a catalytic activity that uses a material as a substrate to the source of the substrate could furnish the substrate at a higher concentration than is present elsewhere in the cell water. It appears that at least the first two of these actually operate in the *aro* complex, and the third cannot be ruled out. The activity of the complex can be assayed by furnishing the input compound for dehydroquinic synthesis, 3-deoxy-D-arabinoheptulosonic acid-7-phosphate, and output can be measured; the individual reactions can be studied by putting in any one of the intermediates; or both may be done at once. Very interestingly, when 3-deoxy-D-arabinoheptulosonic acid-7-phosphate is put in along with other substrates, it is found that all of the reactions but one (shikimate kinase) are affected in kinetic properties in ways that could be expected to give a more efficient catalyst in vivo. While there are some modest effects on V_{max} , the K_m is lowered by 5 to 10 times for two of the enzymes and by about 2 times for two others. Gaertner and co-workers (51, 134, 135) feel that reduction of the K_m at critical points in the pathway is probably the most important factor in the catalytic facilitation. They also have confirmed the essentials of this work with enzyme considered to be free of proteolytic modification (F. H. Gaertner, personal communication). They also feel that the kinetics of shikimate kinase may have a special

role in regulating the sluicing of excess anabolic capacity into catabolism during times of adenosine 5'-triphosphate shortage. (For a discussion of this interesting but complicated hypothesis, see reference 135).

A more sophisticated measure of efficiency of an enzyme complex is the transient time—in this case, the time required to achieve a steady-state rate of output of 5-enol-pyruvyl shikimate-3-phosphate after the reaction is started with 3-deoxy-D-arabinoheptulosonic acid-7-phosphate. This transient time is a measure of the aggregate K_m and V_{max} values of the individual enzymes plus the time it takes for a given amount of each enzyme to build up a steady-state pool of its product—the substrate for the next enzyme. It can be seen that the shortness of the transient time is a sort of figure of merit for an enzyme system. When the kinetic parameters of the individual enzymes, assayed with exogenous substrates, are used with an analog computer to model the output of final product, it is found that these cannot account for the shortness of the transient time seen with the actual complex (134). An attractive interpretation is that the *aro* complex allows biosynthesis of 5-enol-pyruvyl shikimate-3-phosphate without requiring the buildup of large intracellular pools of its precursors. This may be thought of in terms of reducing the need for large pools of osmotically active particles. Also note that the enzyme molecule itself may be thought of as a single osmotically active particle, whereas five separate enzymes would constitute five osmotically active particles. (The significance of this sort of osmotic pressure reduction is more doubtful, as noted above. None of these enzymes is likely to be at its isoelectric point in the cell, and each will probably have a retinue of cations associated with it; these must be thought of as being at least partly osmotically active, so reducing the number of polypeptide chain without reducing the number of associated cations may have a rather minor effect.)

There are two cases of enzymatic pinch-hitting between the *aro* system and the *qa* system that are so striking as to invite interpretation. First of all, the anabolic dehydroquinase is, at least to a first approximation, a dispensable enzyme, since, if it is missing, anabolically formed dehydroquinic acid induces the *qa* enzyme system and the resulting catabolic dehydroquinase can function in an anabolic role. Indeed, mutants lacking anabolic dehydroquinase can only be isolated in a strain in which the *qa* system has been mutationally disrupted—for example, by a *qa-1* mutation (111). Why should there be two different dehydroquinases? Since enzymes do

not affect the equilibrium of a reaction, the answer must be sought in terms of control mechanisms, or in terms of enzyme kinetics. The former is obvious, but let us look at the latter. There may be a general kinetic explanation of why it is useful to have both an anabolic enzyme and a catabolic enzyme. The Briggs-Haldane equation states that $[(V_{max})_{forward}/(V_{max})_{reverse}] \times [(K_m)_{reverse}/(K_m)_{forward}] = K_{eq}$, where (V_{max}) is the maximum velocity in the direction noted and K_m is the Michaelis constant. (The possible selective importance of V_{max} and K_m has been considered from a somewhat different viewpoint by others [see, for example, Atkinson (3) and references in that article].) For a catabolic enzyme that must furnish carbon and energy to all other pathways, a high V_{max} is essential. On the other hand a low K_m value may be quite unimportant, because the pathway would not be useful in the first place unless the substrate in question were abundant. But the equilibrium constant, K_{eq} , is not subject to evolutionary change, and therefore a very high $(V_{max})_{forward}$ can only be obtained by adjusting the other kinetic constants. It is reasonable to argue that part of the price of a very high $(V_{max})_{forward}$ will be paid in terms of a high $(K_m)_{forward}$ value. Conversely, only a very small fraction of the carbon flow of the cell will be through the pathway of aromatic biosynthesis, so a very high $(V_{max})_{forward}$ is less important.

In the case of anabolism, it is obvious that if each of hundreds of different anabolic intermediates were allowed to build up to a substantial concentration, the total osmotic pressure would be intolerably large. Thus, a low $(K_m)_{forward}$ value will be very advantageous in anabolic pathways. This can be paid for with a relatively low $(V_{max})_{forward}$, since only a very small part of the flow of carbon in a cell goes through any one anabolic pathway. If we compare the $(K_m)_{forward}$ values for the catabolic and anabolic dehydroquinases, we see that, indeed, the catabolic enzyme has a much higher K_m value than does the anabolic enzyme (Table 1). The relative V_{max} values for the catabolic and anabolic enzymes shown in Table 1 also differ in the expected direction. This should not be overinterpreted, however. First of all, the values, expressed here in terms of activity per milligram of true protein mass, reflect only dehydroquinase activity in the case of the catabolic enzyme, but they include amino acid sequence devoted to four other enzymatic activities in the case of the anabolic enzyme. In addition, the two values I have calculated from the data of two different laboratories are probably in fortuitously close agreement, in view of the differences in mode of preparation

TABLE 1. Kinetic constants of catabolic and anabolic dehydroquinases (expressed for forward, or hydration, reaction)

Enzyme	Kinetic parameter (reference)	
	K_m (mM)	V_{max} (μ mol/min per mg)
Catabolic	0.36 (Rines ^a)	255 (66) ^b
Anabolic	0.02 (135; Rines ^a) ^c	9.2 (49) ^d
	0.1 (135) ^c	10.9 (86) ^f

^a H. W. Rines, Genetics 60:215, 1968; H. W. Rines, Ph.D. thesis, Yale University, New Haven, Conn., 1969.

^b Most active fractions, assayed at 37°C. The specific activity reported here is 1,000 times higher than that recorded by Hautala et al. (66); in that publication, the units were inadvertently noted as nanomoles per minute instead of micromoles per minute (J. Hautala, personal communication).

^c Enzyme activated by 3-deoxy-D-arabinoheptulosonic acid-7-phosphate.

^d Enzyme assayed at 25°C, pH 7, with V_{max} for dehydroquinase calculated from the ratio of activity of that enzyme given by Welch and Gaertner (135), and 1 mg of protein per ml taken as 0.68 absorbancy at 280 nm (86).

^e Enzyme not activated.

^f Enzyme assayed at 37°C, pH 10.6.

and assay conditions and the wide divergence between the values for other activities in the enzyme complex. Even with these reservations, the differences between the catabolic and the anabolic enzymes are of such a magnitude that it is hard to explain them away.

A second intriguing case of substitution of function involves quinate dehydrogenase, the enzyme coded by *qa-3*. Like other catabolic dehydrogenases, it is an oxidized nicotinamide adenine dinucleotide (oxidized NAD)-utilizing enzyme. It will accept shikimic acid as a substrate for dehydrogenation, though this is probably a gratuitous reaction in a normal cell. However, in a cell that has lost its anabolic dehydroshikimate reductase (shikimate dehydrogenase) through an *aro-1* mutation, the catabolic enzyme can function under certain circumstances in the reverse of its normal direction, to convert accumulated dehydroshikimic acid to shikimic acid, using reduced NAD plus H^+ as a reducing agent (the anabolic enzyme uses reduced NAD-phosphate plus H^+ as a reducing agent [reduced NADP]). What is remarkable about this is its discordance with a major generalization on the energetics of anabolism and catabolism. This is that anabolic pathways almost always used reduced NADP as the reducing agent, whereas catabolic pathways used oxidized NAD as the oxidizing agent. At least part of the thermody-

namic drive for anabolism comes from the fact that the intracellular reduced NADP-oxidized NADP ratio is maintained at a high level, favoring anabolism, whereas the reduced NAD-oxidized NAD ratio is kept low, favoring catabolism (for ratios in rat liver, see reference 78). These biased molar ratios also obtain in *Neurospora*, though to a less dramatic degree (19). Although the free energy available from a high reduced NADP-oxidized NADP ratio no doubt favors reduction of dehydroshikimic acid, it is not obligatory, since reduced NAD and an NAD-specific enzyme can substitute for it in an anabolic capacity. As a final note, it is interesting that there is a parallel case in *Neurospora* in which such a substitution is also possible (on paper), but does not operate physiologically. There are two glutamate dehydrogenases in *Neurospora*. An NADP-specific enzyme catalyzes the reduction of ammonium ion plus α -ketoglutaric acid to give glutamic acid in the net assimilation of inorganic nitrogen (44). An NAD-specific enzyme catalyzes nominally the same reaction, but it probably operates physiologically in the direction of glutamic acid catabolism, since it is induced by the presence of glutamic acid in the medium (114). Mutants missing the anabolic enzyme are nevertheless auxotrophs. Hence, it seems that some reactions do not require the thermodynamic drive of a high reduced NADP-oxidized NADP ratio, but others do.

Phosphorus Acquisition System

The normal media used for growth of *Neurospora* all contain excess phosphorus in the form of inorganic phosphate. When the mold is instead grown with severely limited inorganic phosphate or on a rate-limiting phosphorus source (phosphorylethanolamine), some seven enzymes are formed which occur only in much smaller amounts, if at all, in phosphorus-sufficient cultures. Each of these enzymes (the list is no doubt incomplete) is concerned in some obvious way with garnering phosphorus for the cell in times of dearth. The enzymes are: a repressible alkaline phosphatase (24, 75, 100a, 127), a repressible acid phosphatase (80, 100, 127), a 5'-nucleotidase (64), three nucleases (63, 65), a repressible phosphate permease (80, 84; M. S. Lowendorf and C. W. Slayman, *Bacteriol. Proc.*, p. 130-131, 1970), and a phosphorylethanolamine-phosphorylcholine permease (R. L. Metzberg, unpublished data). Most of these enzymes have counterparts that are made even in phosphate sufficiency. I will refer to these as "housekeeping enzymes" to avoid calling them "constitutive," a term I will reserve for the repressible enzymes when they appear in mutants

grown under conditions that normally cause repression. The housekeeping enzymes have different molecular properties from the repressible ones. In the case of alkaline phosphatases, there are major differences in isoelectric point, stability, metal requirement, and other properties. The housekeeping and the repressible forms are not immunologically cross-reactive (81). In addition, the repressible alkaline phosphatase is almost entirely confined to the space between the cell wall and the plasma membrane, outside the major permeability barrier of the cell, whereas the housekeeping enzyme is, in every sense, inside the cell (24). Taken with the fact that *Neurospora* seems unable to transport any known phosphate esters into the cell other than phosphorylethanolamine and phosphorylcholine, the localization suggests strongly that the housekeeping alkaline phosphatase serves the internal economy of the cell, probably in ways unrelated to phosphorus nutrition. The repressible enzyme in all likelihood acts by splitting phosphate esters present in the medium, with the phosphate then being transported by the (high affinity) repressible phosphate permease (80). If one supposes that a major source of phosphorus in the environment of *Neurospora* will be nucleic acids released from heat-killed plant cells, one is led to postulate three steps involved in acquiring phosphorus from a phosphate-limited medium. There are (i) hydrolysis of nucleic acids to phosphomonoesters by phosphate-repressible nucleases (65), (ii) hydrolysis of these esters by repressible acid phosphatase and 5'-nucleotidase (mostly in the medium [see references 100 and 64] and repressible alkaline phosphatase (mostly in the periplasm [24]), and (iii) transport of the phosphate into the cell. The housekeeping phosphate permease is adequate to transport phosphate into the cell under conditions of phosphate sufficiency and a pH below about 7. The K_m value of this permease, even at low pH, is much higher than that of the repressible phosphate permease. Furthermore, it rises rapidly with pH, so that at a pH of greater than 7 and external phosphate concentrations of 50 μ M or less, the repressible enzyme is absolutely required for growth (80, 84). This property is readily used as the basis of both scoring and selection of mutants.

The range of expression of the genes for these enzymes, particularly of the repressible alkaline phosphatase, is very large. Under conditions of phosphate starvation, the amount made is at least 10,000 times that in a phosphate-sufficient culture, though to demonstrate the true low value of the repressible enzyme in phosphate sufficiency, it is necessary to physically remove

the housekeeping enzyme (81). The repressible phosphate permease may have a much narrower range of expression than other genes of the family, however (12a).

The maximum commitment of resources to this phosphatase has been estimated in a phosphate-sufficient culture of a recently discovered constitutive mutant called *pgov*^{c-12}. Immunoprecipitation of pulse-labeled enzyme indicates that 4.7% of the soluble protein being synthesized is repressible alkaline phosphatase (S. J. Free, personal communication). The specific activity achieved is similar to that of the wild type grown under conditions of phosphorus starvation. If the other enzymes of the family are made in anything like similar amounts, this must represent a major redirection of protein synthesis into untimely formation of these enzymes. The fact that *pgov*^{c-12} and other intensely derepressed strains also grow poorly suggests that this wastefulness is costly (Metzenberg, unpublished data).

A question that must immediately arise about such a system is whether the structural genes for the family of enzymes are clustered, as in the case with the *qa* and *aro* systems. Structural gene mutants that alter or destroy the activity of two individual enzymes are known. *pho-2* mutants generally are devoid of repressible alkaline phosphatase activity, though some produce immunologically cross-reacting material (59, 81). One unusual allele, *pho-2(MKG-2)* is capable of producing about 1% of the activity of the wild type, rather than none at all. The small amount of enzyme made by this strain seems to be identical in quality with that of the wild type. *pho-2(MKG-2)* maps very close to other *pho-2* alleles, and it is suggested that it defines a regulatory locus, in this case, the target for a positive-acting protein produced by the *nuc-1* gene (see below).

The *pho-3* locus, which is the structural gene for repressible acid phosphatase, is identified by a single mutation. The mutant strain produces a temperature-sensitive repressible acid phosphatase that also has an altered *K_m* (96). *pho-2* and *pho-3* are unlinked to one another and to the known regulatory genes (see below). Although it is not impossible that some clustered structural genes will be found, there is no indication of it at present.

The regulatory system as revealed by the mutants is extraordinarily complicated. There are four genes involved: *nuc-1* and *nuc-2* (82, 127), *preg* (82), and a gene provisionally called *pgov* (Metzenberg, unpublished data; and see above). Two kinds of mutations are known at the *nuc-1* locus: null mutants called *nuc-1*, which fail to make any of the enzymes of the phosphorus

family, and *nuc-1^c* mutants, which are constitutive for at least those enzymes that have been examined, and presumably for the rest. *nuc-1* mutants are recessive to the wild-type allele, *nuc-1⁺*, both in heterocaryons and in partial diploids; *nuc-1^c* is dominant to both *nuc-1⁺* and *nuc-1* in partial diploids, but it is dominant in heterocaryons only when a fairly large proportion of the nuclei are *nuc-1^c* (W. Chia, M. S. thesis, University of Wisconsin, Madison, 1976; R. L. Metzenberg and W. Chia, Genetics, in press; for discussion, see below). There may, in fact, be a third kind of mutant allele at *nuc-1*. Gleason and Metzenberg (59) found a suppressor of *pho-2(MKG-2)* that raised the level of expression of this mutant from about 1% of normal to about 40%. The suppressor had no recognizable phenotype except through its effect on *pho-2(MKG-2)*. It was found to lie close to *nuc-1*. Because of the difficulties of mapping from crosses in which such a conditional phenotype had to be scored, it was not possible to set limits closer than about 1 to 2 centimorgans, but it is quite possible that the suppressor maps in *nuc-1*.

Mutants at *nuc-2* are of two kinds: *nuc-2* null mutants, which are phenotypically indistinguishable from *nuc-1*, though they map on a different linkage group, and *pcon^c* (phosphorus control constitutive) mutants. The terminology for these was unfortunately chosen before it was appreciated that these were allelic with *nuc-2* mutants (82). *nuc-2* mutants are recessive to *nuc-2⁺* in both heterocaryons and in partial diploids, and *pcon^c* ("*nuc-2^c*") mutants are dominant to *nuc-2⁺* and to *nuc-2* in both heterocaryons and in partial diploids (93).

The *preg* (phosphorus regulation) locus is identified only by the occurrence of constitutive mutants, not null mutants. Unlike the two types of constitutive mutants just described, these are completely recessive to their wild-type allele in heterocaryons and in partial diploids (93). *preg^c* mutants map about 1 to 2 centimorgans from *nuc-2*. The two genes behave as separate cistrons. The linkage between them seems too close to be fortuitous (the genome of *N. crassa* is estimated at about 1,000 centimorgans [103]). On the other hand, this crossover distance is at least an order of magnitude greater than is usually seen for contiguous genes in *Neurospora*. The significance of the close linkage is not known. The remaining locus, *pgov* (phosphorus governance) also has given rise thus far only to recessive constitutive mutants (*pgov^c*). These mutants seemingly are much rarer than *preg^c* mutants and have only been isolated in *preg⁺/preg⁺* partial diploids, in which *preg^c* mutants

are not detected. The *pgov^c* mutants can then be extracted from the aneuploid background. *pgov*, *nuc-1*, *nuc-2*, *preg*, *pho-2*, and *pho-3* are on five different chromosomes of the seven in the *Neurospora* genome, a situation hardly reminiscent of the classical lactose operon.

It is almost impossible to engage this tangle of facts without a model. One would hope to construct a model that accurately reflects physical reality, but even one that merely gives some conceptual pegs to hang the facts on is likely to be useful. In making such a model, the epistasis-hypostasis relationships between mutants at these loci have proved to be even more useful than the dominance-recessiveness relationships between alleles at a locus. In an epistasis test, one takes two mutants with contrasting phenotypes that map in two different genes and makes the double mutant. The one that "wins" is epistatic. When this is done with *nuc-1* and either *pcon^c* ("*nuc-2^c*"), *preg^c*, or *pgov^c*, it is found that *nuc-1* is completely epistatic—the double mutants have the same phenotype as *nuc-1*. Conversely, when the double mutant between *nuc-1^c* and *nuc-2* is made, it is constitutive. Hence, both types of mutants at the *nuc-1* locus are epistatic to all others, and it seems clear that the product of this locus has the last word on whether the structural genes will be expressed or not. In an analogous way, we find that *preg^c* is epistatic to *nuc-2*, the double mutant being fully as constitutive as is *preg^c* alone (82); and *pgov^c* is indistinguishable from *preg^c* in this respect (Metzenberg, unpublished data). It is interesting that *nuc-1* and *nuc-2*, which are both null mutants with identical phenotypes, have diametrically opposite phenotypes when viewed in a background of either *preg^c* or *pgov^c*. Finally, it is necessary to assign roles to these elements and to phosphate or its derived corepressor. *nuc-1⁺* is viewed as a positive control substance that is necessary for the expression of the structural genes, control being probably exerted at the level of transcription (M. K. Gleason, M.S. thesis, University of Wisconsin, Madison, 1973). The *preg⁺* product is imagined as opposing *nuc-1⁺* either by repressing transcription of the gene or by inactivating or complexing the gene product. (Reasons for favoring the latter hypothesis will be given below.) The *nuc-2⁺* product is viewed as opposing *preg⁺*, again, either by repressing its transcription or by nullifying activity of existing *nuc-2⁺* product. The *nuc-2⁺* product is believed to be the target for inactivation by phosphate or a corepressor derived from it. Finally, the position of the recently discovered *pgov⁺* in the epistasis hierarchy is identical with that of *preg⁺*. One of several possible working

hypotheses is that the two form polypeptides that interact to form the negative controller of the positive expressor, the *nuc-1⁺* product. The hierarchy can be diagrammed as shown in Fig. 4. (*pgov* is omitted in the interest of clarity.) This very complicated control system bears some strong formal similarities to the genetic elements that control acid phosphatase in yeast (129).

The properties of one *nuc-1^c* allele that has been examined in more detail allow some additional hypotheses to be made about the nature of both *nuc-1^c* and the role of *preg⁺*. *nuc-1^c* can be put in a genetic background that is diploid for a short region including the *nuc-2* and *preg* genes, using combinations of alleles of these two genes (94). Consider the simple case of strains that are homozygous for lack of *nuc-2* function but which carry either zero, one, or two doses of functional *preg⁺* or, respectively, two, one, and zero doses of *preg^c* (Table 2, strains C, F, and I). When three such strains are grown on high phosphate, the specific activities of repressible alkaline phosphatase are, respectively, 766, 297, and 1.22. The last of these values corresponds to essentially full repression, given that no special pains were taken to remove the housekeeping enzyme. There is not only an obvious difference between zero and one dose of *preg⁺*, there is an even more significant difference between one and two doses. Under these circumstances, should we say that *preg^c* is recessive? In a *nuc-1⁺* background it is; in a *nuc-1^c* background it is not. To illustrate this, we can conjure up a world

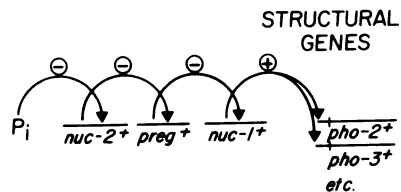


FIG. 4. Model for the control of synthesis of the phosphorus family of enzymes. *pho-2*, *pho-3*, etc., are the structural genes, which are under positive control by the product of the *nuc-1⁺* product. This product is inactivated by the *preg⁺* product, which in turn is inactivated or repressed by the *nuc-2⁺* product, which in turn is inactivated or repressed by phosphate (P_i) or something derived from it. The role of the *pgov⁺* gene product (not shown) is identical with that of the *preg⁺* product. The phenotypes of various strains carrying one or more mutations can be calculated by multiplying the positive or negative signs of the regulatory products. Only those regulatory products that are connected to the structural genes by a sequence unbroken by mutation can be included in the calculation. For example, in *nuc-2* strains, only the final two regulatory steps are operative ($- \times +$) and such strains are $-$, i.e., the enzymes are not made.

TABLE 2. Gene dosage effects on synthesis of alkaline phosphatase in *nuc-1^c*^a

Strain	Allele at <i>nuc-1</i>	Normal sequence, allele at:		Translocated segment, allele at:		Gene dosage of:		Alkaline phosphatase sp act with:	
		<i>nuc-2</i>	<i>preg</i>	<i>nuc-2</i>	<i>preg</i>	<i>nuc-2⁺</i>	<i>preg⁺</i>	Phosphoethanolamine, 2 mM	Inorganic phosphate, 7.35 mM
A	c	+	c	+	c	2	0	1,482	873
B	c	Null	c	+	c	1	0	1,060	923
C	c	Null	c	ts	c	0	0	800	766
D	c	+	+	+	c	2	1	292	293
E	c	Null	+	+	c	1	1	307	268
F	c	Null	+	ts	c	0	1	252	297
G	c	+	+	+	+	2	2	144	34.9
H	c	Null	+	+	+	1	2	133	14.3
I	c	Null	+	ts	+	0	2	36.1	1.22
J	c	+	+	Not present		1	1	186	150
K	c	Not present		+	c	1	0	641	801
L	+	+	+	Not present		1	1	100	1.08

^a Strains A through I are partial diploids carrying a duplication of the *nuc-2* and *preg* region of linkage group II. Strains J and L are euploid and have the normal sequence of genes. Strain K is euploid and has the *nuc-2* to *preg* region of linkage group II translocated to linkage group I. ts is a temperature-sensitive *nuc-2* mutant. All the strains were grown at 35°C, a temperature which is restrictive for ts. (Abridged from Metzenberg and Nelson, [94].)

in which the wild type is aneuploid and repressible and has the constitution *nuc-1^c*; *nuc-2⁺ preg⁺/nuc-2⁺ preg⁺*. A mutation of one of the *preg⁺* alleles to *preg^c* makes the strain constitutive (a "mutation" from strain G to strain D). Under these conditions, *preg^c* mutants would have been identified as dominant.

It is clumsy, if not quite impossible, to explain the phenotype of *nuc-1^c* by postulating that *preg⁺* normally turns off the synthesis of the *nuc-1⁺* product but that *nuc-1^c* is an operator mutant which cannot be repressed. This would require the rationalization that the operator of *nuc-1^c* is blind to one dose of *preg⁺*, or nearly so, but that it sees two doses of *preg⁺* very well. Another hypothesis, that *nuc-1^c* makes a special product that has lost its binding site for the *preg⁺* product, suffers from the same inconsistency. A third hypothesis, one consistent with the facts, is that the *preg⁺* product normally acts by titrating out the *nuc-1⁺* product unless the *preg⁺* product has been negated by *nuc-2⁺* product. *nuc-1^c* can be viewed as an overproducer of the qualitatively normal *nuc-1* product such that the usual haploid level of the *preg⁺* product is insufficient to titrate it out but that two doses does so effectively. It is helpful in thinking about this provisional model to assign some arbitrary numbers to the amounts of the *nuc-1* and *preg* products. Suppose that the wild type, *nuc-1⁺*, gene makes 100 arbitrary units of product, that *nuc-1^c* makes 250 units of the same material, and of course, that *nuc-1* makes none. Let us suppose that *preg⁺* makes 150 units of the *preg* product and that *preg^c* makes none. We must assume

that the *preg⁺* product is only made, or is only effective, when inorganic phosphate is in adequate supply, since in the absence of phosphate it is counteracted by the normal *nuc-2* product (see Fig. 4). If 150 units of the *preg* product are present and active, no *nuc-1⁺* will be left over in the wild type, and no expression of the phosphate family or genes will occur. If the allele at *nuc-1* is *nuc-1^c* so that there are 250 units of this product, a *preg⁺* gene making 150 units will not be able to complex it all out, and the mutant will be classified as constitutive; but the partial diploid *nuc-1^c; preg^{+/+} (nuc-2^{+/+})* will, under conditions of phosphate adequacy, have 300 units of the *preg* product, and the strain will be repressible.

This dosage titration model also makes some distinctive predictions about the behavior of heterocaryons between mutants in the *nuc-1* locus. If one assumes that the excess *nuc-1* product of *nuc-1^c* is diluted in the cytoplasm of a heterocaryon with either *nuc-1⁺* or *nuc-1*, these heterocaryons should be constitutive if *nuc-1^c* nuclei were present in high proportion but repressible if they were present in low proportion. On the other hand, partial diploids heterozygous for *nuc-1^c* and either *nuc-1⁺* or *nuc-1* should simply be constitutive. These predictions are borne out (Metzenberg and Chia, Genetics, in press).

Obviously, dominance and recessiveness lose their usual meanings when one may be dealing with a situation in which the product of one gene may react stoichiometrically with the product of another. One is prompted to wonder if some of the deleterious effects of aneuploidy for human

autosomes should be considered in terms of altered stoichiometry between regulatory proteins.

The molecular nature of the four regulatory substances corresponding to *nuc-1⁺*, *preg⁺*, *pgov⁺*, and *nuc-2⁺* remain completely unknown. It is attractive to think of the *nuc-1⁺* gene as making a protein that binds to a target site next to each of the structural genes. The presence of this protein would be necessary to activate transcription of the structural genes of the family. Such a protein could work solely on the DNA of the target site, or it could take part in a concerted action with the site and RNA polymerase. In either case, one would expect to find it in the nucleus. There is no evidence on how the other regulatory molecules interact with one another. It is perfectly possible that all of them occur together physically as a complex, perhaps much like the glutamine synthetase regulatory complex of enterobacteria (52, 58, 87) or the glycogen phosphorylase system of mammals (46). It remains to be seen whether multigenic control systems generally will operate through interacting gene products which are themselves made constitutively or whether they will work via a cascade of induction and repression, with the control elements themselves being controlled. Some recent findings indicate that the products of regulatory genes for the galactose system of yeast are made constitutively (91, 106).

Alternative Modes of Expression of an Extracellular Protease

Neurospora must frequently encounter proteins in exudates of charred plant material, and, in some cases, protein probably constitutes the sole significant source of either carbon, nitrogen, or sulfur. It is to be expected that the pH in such a habitat will often be above 7.0 due to combustion of the sodium and potassium salts of organic acids of plant cells (alkali = Arabic *al qaliy*, "the ashes"). It is not surprising, then, to find that *Neurospora* is capable of making and secreting several proteases, in particular, one that is both stable and active at elevated pH. What is unusual is the mode of control. Production of the alkaline protease requires the presence of an extracellular protein source as inducer, plus any one of three different situations of nutritional deprivation. These can be carbon starvation (40), nitrogen starvation (33a, 61a), or sulfur starvation (61a). Later, it was found (62) that the alkaline proteases secreted under these three conditions were indistinguishable in all biochemical properties studied, and Cohen et al. (34) reported that the alkaline proteases made under the different starvation conditions were immu-

nologically cross-reactive. Hanson and Marzluf (62) were not able to select mutants in the putative structural gene, but they did find among a collection of natural isolates of *Neurospora* one which had a protease of altered electrophoretic mobility. The alteration proved to be the same under all three starvation regimens. This strongly supports the idea that any one of three different signals is sufficient to turn on expression of a single gene.

In the case of both nitrogen acquisition and sulfur acquisition, substantial information is available about element-specific control genes, and the role of these genes in control of the alkaline protease is revealing. Ammonium ion seems to be the preferred nitrogen source, and it can repress the formation of a large number of permeases and intracellular enzymes that lead to acquisition of ammonia from nitrate ions, purines, or exogenous amino acids (109, 110, 120; see, however, Tsao and Marzluf [128]). None of these enzymes, including even one permease that is not ammonia repressible, can be formed in anything approaching normal derepressed amounts in the absence of a functional product of the gene *amr* (ammonia regulation [see reference 110]). *amr* is almost surely allelic with the long-known *nit-2*, a gene whose wild-type product is necessary for formation of nitrate reductase. *amr⁺* is envisioned as exerting direct, positive control over the expression of at least some of these genes related to nitrogen acquisition and either direct or indirect control over the rest of them (127). At least four of the activities under the control of *amr* have been mapped to specific genes, and these are unlinked or weakly linked to *amr* and to one another. The important point to be made here is that the *amr⁺* product is needed not only for the synthesis of these enzymes of nitrogen acquisition but also for synthesis of the alkaline protease—but only under conditions of nitrogen starvation. The enzyme can be made perfectly well in *amr* mutants under conditions of carbon or sulfur starvation.

Just as lack of ammonium ions seems to be the cue for production of the nitrogen family of enzymes, lack of intracellular cysteine is apparently the signal to make enzymes of sulfur acquisition (72, 77). Growth on a rate-limiting sulfur source, such as cysteic acid, or simple sulfur starvation results in the synthesis of a family of enzymes made in small or undetectable amounts in sulfur-sufficient cultures (23, 90). Two genes, *cys-3* and *scon*, exert a high degree of control over the formation of these enzymes. *cys-3* mutants are pleiotropic null mutants for formation of all of the enzymes, whereas the sole *scon⁺*

mutant is constitutive for all of them and has unusual properties that are outside the scope of this review (23). *cys-3* is epistatic to *scon*^c in double mutants (39) and is believed to exert positive control over the expression of structural genes for the sulfur family of enzymes. As with the phosphorus and nitrogen families, the several known structural genes for enzymes are unlinked or distantly linked to the control genes and to one another (88, 92). *cys-3* seems analogous with *amr* and with *nuc-1* in the phosphorus control scheme. As might be guessed from the analogy with *amr* mutants, *cys-3* mutants are unable to make the alkaline protease under conditions of sulfur starvation, but they do so normally under conditions of carbon or nitrogen starvation.

We do not know whether the symmetry is complete—whether there is a carbon family gene necessary for the expression of alkaline protease during carbon starvation, but the notion is certainly attractive. Hanson and Marzluf (62) have suggested a model, shown very slightly modified in Fig. 5, in which alternate positive control signals turn on expression of the alkaline protease gene by binding to one of three receptors adjacent to the gene. They suggest that glucose or cyclic adenosine 3',5'-monophosphate is a likely candidate for the carbon corepressor analogous to ammonium ion and cysteine. It would surely be of interest to identify a gene that allows poor carbon sources to be harvested from the medium and used when glucose or cyclic adenosine 3',5'-monophosphate levels in the cell are low. A reasonable way to look for such a gene is by selecting mutants that can grow on glucose but that are simultaneously unable to make several disaccharases, such as β -fructofuranosidase, maltase, and α , α -trehalase, and therefore cannot grow on the corre-

sponding sugars. As yet, no such mutants have been reported, and it will be interesting to see if future work is successful in turning them up. Mutants with drastically reduced levels of cyclic adenosine 3',5'-monophosphate (112, 117, 118) and of adenylate cyclase (47) are known, and these promise to be of interest in studying the response to carbon starvation.

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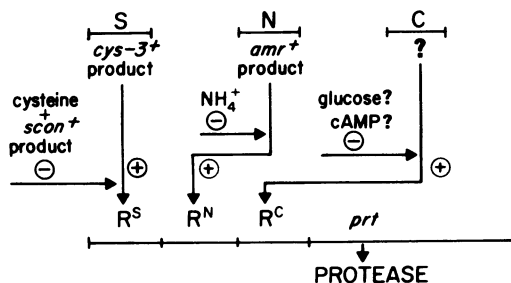


FIG. 5. Model for the control of synthesis of alkaline protease. The structural gene, *prt*, is envisioned as being under the control of any one of three cis-acting sites, R^S , R^N , or R^C , each of which is responsive to a positive-acting, diffusible regulator. Each of these regulators can be nullified by the appropriate corepressor. For further description, see the text.

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